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Amino Acid Composition of β-Lactoglobulins A, B, and AB

It has been reported that the genetically different β-lactoglobulins A and B discovered by Aschaffenburg and Drewry (1, 2) differ in content of aspartic acid, glycine, alanine, and valine (3). Further data in this connection, as well as the results of some new analyses of β-lactoglobulin AB, are presented here.

While our research was in progress, similar but completely independent work was being carried out by Piez, Davie, Folk, and Gladner[1] at the National Institutes of Health. It is of particular interest that the β-lactoglobulins A and B analyzed by these investigators were prepared by chromatographic resolution of β-lactoglobulin AB, whereas our analyses of the two forms of the protein were run on samples crystallized from typed milks. Nevertheless, the two sets of analyses are in agreement with respect to the significant differences in amino acid composition.[1]

On comparison of the early analyses of β-lactoglobulin by Brand *et al.* (4) and the somewhat later ones by Stein and Moore (5) with the present results, it is clear that only minor revisions in the amino acid composition of this protein need be proposed.

EXPERIMENTAL PROCEDURE

β-Lactoglobulins A and B were crystallized by the method of Aschaffenburg and Drewry (6) from typed milks.[2] β-Lactoglobulin AB was prepared from mixed herd milk by Palmer's method (7) with ammonium sulfate for the fractionation of whey proteins (8). β-Lactoglobulins A and B were recrystallized three times and lactoglobulin AB five times before final dialysis and lyophilization.

Weighed samples of the proteins were hydrolyzed at 110° (oil bath) in a 200-fold quantity of glass-distilled 6 N HCl in sealed, evacuated tubes.[3] Periods of 24, 72, and 96 hours were employed for hydrolysis.

The amino acid analyses were done according to the procedure of Spackman, Stein, and Moore (9) in a Phoenix model K-5000 amino acid analyzer.[4]

RESULTS

The analytical results are presented in Table I. It is apparent that most of the amino acids occur in equal concentration in β-lactoglobulins A and B and, therefore, in the mixed lactoglobulin AB as well. However, β-lactoglobulin A contains more aspartic acid and valine, but less glycine and alanine than the B form, whereas β-lactoglobulin AB contains intermediate amounts of these amino acids. That these differences between A and B are indeed significant is borne out by statistical analysis of the results.[5]

The significant difference in valine content may be seen not only in the final values obtained from the 96-hour hydrolysates but also in the figures in parentheses, the results from 24-hour hydrolysates. The constancy of the isoleucine values is apparent as well at both time periods.

Any significance which might be attached to the differences in threonine and serine content, indicated by the final values in Table I, would be doubtful because these are extrapolated values. The analyses of the 24-hour hydrolysates show both threonine and serine to be somewhat higher in A than in B, whereas after 96 hours of hydrolysis they are higher in B. Obviously, unequivocal conclusions cannot be drawn from these data. Likewise, any inferences regarding amide groups, suggested by the similarity in ammonia values for both 24- and 96-hour hydrolysates of A and B, would be of dubious validity.

The results for cystine may be disregarded because of variable destruction during hydrolysis. The accepted value for the total cystine plus cysteine content of β-lactoglobulin is 3.40% and that for its total sulfur content 1.60%, as determined by Brand *et al.* (4). The total sulfur contents of β-lactoglobulins A and B are 1.59 and 1.57%, respectively.[6] Because the methionine analyses in Table I show so little variation, it is probable that the cystine plus cysteine content of these proteins is the same.

Analyses of the β-lactoglobulins for tryptophan by the method of Spies and Chambers have been published previously (10); no

* Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

[1] K. A. Piez, E. W. Davie, J. E. Folk, and J. A. Gladner, Personal communications.

[2] We are indebted to R. Townend of this laboratory for making these milks available to us.

[3] A referee has called the attention of the authors to the possible presence of cysteine in the hydrolysates, and to its emergence under proline in the analytical system employed unless it is previously converted to cystine by neutralization of the hydrolysates and exposure to air before analysis. Our hydrolysates were not neutralized, and it is possible, therefore, that the results are somewhat high for proline and too low for cystine.

[4] Mention of specific firms and products does not imply endorsement by the Department of Agriculture to the possible detriment of others not mentioned.

[5] The statistical treatment of aspartic acid, glycine, and alanine was by group comparison instead of by pairing, since there was an odd number of total determinations for each of these amino acids and since time of hydrolysis did not affect these values. However, with valine, for which the values increased with time of hydrolysis, pairing was used; and an unpaired value for A was omitted. The p values were as follows: aspartic acid, <0.001; glycine, <0.001; alanine, 0.004; and valine, 0.001. Values of 0.05 indicate a significant difference between mean values at a confidence level of 95%; values below 0.05 indicate a significant difference at even higher confidence levels. We are indebted to M. L. Groves for this analysis.

[6] We thank Mrs. R. B. Kelly of this laboratory for these determinations.

TABLE I

Amino acid composition of β-lactoglobulins

[1] Figures are in grams of amino acid per 100 g of dry protein.

	Time of hydrolysis							Final extrapolated value or average value[a] with average deviation		
	24 hours			72 hours	96 hours					
	AB	A	B	AB	AB	A	B	AB	A	B
Aspartic acid	11.17	11.40	10.63	11.33	11.14	11.37	10.87	11.22 ± 0.10	11.39 ± 0.12	10.72 ± 0.16
Threonine	4.84	4.90	4.76	4.53	4.49	4.55	4.69	4.94	5.01	4.79
Serine	3.36	3.34	3.22	2.76	2.62	2.69	3.01	3.64	3.58	3.31
Glutamic acid	19.09	19.12	18.74	19.50	19.15	19.12	19.51	19.26 ± 0.24	19.12 ± 0.18	19.05 ± 0.37
Proline	5.04	5.29	5.02	5.01	5.29	5.15	5.18	5.09 ± 0.17	5.22 ± 0.08	5.08 ± 0.07
Glycine	1.38	1.24	1.54	1.43	1.41	1.24	1.58	1.41 ± 0.02	1.24 ± 0.02	1.55 ± 0.02
Alanine	6.94	6.69	7.00	7.06	6.92	6.72	7.08	6.98 ± 0.10	6.70 ± 0.11	7.03 ± 0.10
Cystine	2.0	2.1	2.0	1.8	1.9	2.5	2.5			
Valine	(5.80)	(5.96)	(5.39)	6.07	5.96	6.11	5.72	6.03 ± 0.09	6.11 ± 0.03	5.72 ± 0.07
Methionine	3.16	3.18	3.12	3.19	3.14	3.14	3.20	3.16 ± 0.05	3.16 ± 0.04	3.15 ± 0.04
Isoleucine	(6.15)	(6.08)	(6.14)	6.87	6.93	6.76	6.79	6.89 ± 0.09	6.76 ± 0.11	6.79 ± 0.04
Leucine	15.13	15.13	14.88	15.38	15.17	15.00	15.07	15.23 ± 0.19	15.08 ± 0.15	14.96 ± 0.22
Tyrosine	3.90	3.82	3.80	3.90	3.92	3.94	3.78	3.90 ± 0.03	3.87 ± 0.08	3.79 ± 0.04
Phenylalanine	3.53	3.50	3.48	3.57	3.55	3.58	3.50	3.55 ± 0.03	3.53 ± 0.05	3.49 ± 0.04
	3[b]	3	3	3	2	2 or 3	2			
	2[c]	2	2	1	1	1	1			
Lysine	11.51	12.00	11.72	11.36	11.89	11.79	11.62	11.57 ± 0.22	11.93 ± 0.13	11.68 ± 0.04
Histidine	1.53	1.61	1.55	1.58	1.65	1.67	1.66	1.57 ± 0.04	1.63 ± 0.03	1.59 ± 0.04
Ammonia	1.29	1.30	1.27	1.37	1.49	1.39	1.36			
Arginine	2.77	2.77	2.65	2.76	2.84	2.80	2.78	2.79 ± 0.04	2.78 ± 0.04	2.69 ± 0.07

[a] The numbers in parentheses were not used for the final averages. The extrapolated values for serine and threonine were obtained from straight line plots of the 24- and 96-hour average values for A and B and by the method of least squares from all the data for AB.

[b] The numbers on this line show the number of individual hydrolysates analyzed on the 150-cm columns; the figures above are averages of the results.

[c] The numbers on this line show the number of individual hydrolysates analyzed on the 15-cm columns; the figures below are the results or averages of the results.

significant difference in the tryptophan content of 2.6% was observed.

A comparison of our analyses of β-lactoglobulin AB with the earlier results of Brand *et al.* (4) and those of Stein and Moore (5) by starch chromatography is shown in Table II. The present results agree well in most cases with one or both of the previous analyses, but it is likely that the present values for valine and isoleucine are more nearly correct because of the longer period of hydrolysis now used. However, our serine value seems to be unaccountably low. The true tryptophan content of β-lactoglobulin remains uncertain even now. The value of 2.62% was obtained on the intact protein whereas the figure of 1.94%, reported by Brand *et al.*, resulted from careful determinations on alkaline hydrolysates. This point will be further discussed below. Whatever the differences in the analyses listed in Table II, they appear to be completely unrelated to the differences in composition between β-lactoglobulins A and B.[7]

Some calculations of minimal molecular weight based on the analytical results are summarized in Table III. In evaluation of such calculations for a molecule of this size, the accuracy of the analytical method, ±3%, becomes the limiting factor. Only the results for the 6 amino acids present to the extent of 8 residues or less per molecule and, in addition, the accurately determined total sulfur figures were used in computing the average molecular weight, 37,700. It was assumed that each molecule of β-lactoglobulin A and B is made up of identical halves so that even numbers of residues were used in calculating the molecular weights from the minimal molecular weights. Because neither tryptophan figure in Table II fitted in with this assumption, tryptophan analyses were not used in arriving at the average molecular weight. The further assumption was made that the two forms of β-lactoglobulin have almost the same molecular weight; and the final figure of 37,700, therefore, is the mean of two averages. It was thought of interest to include in the table the results of similar calculations for aspartic acid, alanine, and valine even though these are present in large concentration. Similarly, calculated numbers of residues per 37,700 are shown for all other amino acids, although the figures can only be approximate.

It can be seen that the calculated numbers of residues for the first seven determinations agree fairly well with the assumed whole numbers, except in the case of total sulfur where the usual rounding off would give odd whole numbers. The main purpose of these calculations was to arrive at some molecular weight based on the analytical results and then to define the differences

[7] In the last column of Table II, the analyses of Piez *et al.* (see footnote 1) of their preparations of β-lactoglobulins A and B have been included. We have converted their results into grams per 100 g of protein in order to facilitate comparison with the other analyses. We thank Dr. Piez and his colleagues for allowing us to use their data for this purpose.

TABLE II

Comparison of some analyses of β-lactoglobulin AB

	Brand *et al.* (4)	Stein and Moore (5)	This paper	Piez *et al.*[1] A	Piez *et al.*[1] B
	g amino acid/100 g protein				
Aspartic acid	11.4	11.52	11.22	11.70	11.04
Threonine	5.85	4.92	4.94	5.29[a]	
Serine	5.0	3.96	3.64	4.01	
Glutamic acid	19.5	19.08	19.26	20.20	
Proline	4.1	5.14	5.09	5.20	
Glycine	1.4	1.39	1.41	1.25	1.66
Alanine	6.2	7.09	6.98	6.78	7.31
Half-cystine	3.40			3.34[b]	
Valine	5.83	5.62	6.03	6.38	5.76
Methionine	3.22		3.16	3.08	
Isoleucine	8.4	5.86	6.89	7.00	
Leucine	15.6	15.50	15.23	15.79	
Tyrosine	3.78	3.64	3.90	3.55	
Phenylalanine	3.54	3.78	3.55	3.48	
Lysine	11.4	12.58	11.57	11.97	
Histidine	1.58	1.63	1.57	1.58	
Arginine	2.88	2.91	2.79	2.73	
Tryptophan	1.94		2.62[c]	2.82	

[a] The single figures listed in this column represent simple averages of the almost identical results from the β-A and β-B forms.

[b] This figure includes cysteine.

[c] Previously determined (10).

TABLE III

Calculation of molecular weights of β-lactoglobulins

	Minimal molecular weight A	Minimal molecular weight B	Assumed No. of residues A	Assumed No. of residues B	Calculated molecular weght A	Calculated molecular weght B	Calculated No. of residues per average molecular weight 37,700 A	Calculated No. of residues per average molecular weight 37,700 B
Total sulfur	2,020	2,040	18	18	36,300	36,800	18.7	18.5
Histidine	9,520	9,760	4	4	38,100	39,000	4.0	3.9
Arginine	6,270	6,480	6	6	37,600	38,900	6.0	5.8
Phenylalanine	4,680	4,730	8	8	37,400	37,900	8.1	8.0
Tyrosine	4,680	4,780	8	8	37,500	38,200	8.1	7.9
Methionine	4,720	4,740	8	8	37,800	37,900	8.0	8.0
Glycine	6,050	4,840	6	8	36,300	38,700	6.2	7.8
					37,300[a]	38,200[a]		
Aspartic acid	1,170	1,240	32	30	37,400	37,300	32.3	30.4
Alanine	1,330	1,270	28	30	37,200	38,000	28.4	29.7
Valine	1,920	2,050	20	18	38,400	36,900	19.7	18.4
Glutamic acid							49.0	48.8
Leucine							43.3	43.0
Lysine							30.8	30.0
Isoleucine							19.4	19.5
Proline							17.1	16.6
Threonine							15.9	15.2
Serine							12.8	11.9
Tryptophan							4.9[b]	4.9[b]
Tryptophan							3.6[c]	3.6[c]
Half-cystine							10.7[d]	10.7[d]

[a] Average of 7-figures above.

[b] From value of 2.65%.

[c] From value of 1.94%.

[d] From value of 3.40%.

in amino acid composition in terms of residues per mole of protein. Per 37,700 molecular weight, β-lactoglobulin A contains 1.9 more aspartic acid residues, 1.3 more valines, 1.6 fewer glycines, and 1.3 fewer alanines than the B form. Per 35,000 molecular weight, the value generally accepted on the basis of physicochemical measurements (*e.g.* (11)), these figures become +1.8, +1.2 −1.4, and −1.3, respectively.

It is difficult to reconcile either set of figures with the concept, for which there is considerable evidence (11–13), that the molecules of the β-lactoglobulins are made up of identical halves. However, if the figures for glycine, aspartic acid, alanine, and valine in the last two columns of Table III are rounded off to the nearest whole number, which would be permissible considering the accuracy of the analytical method, each number would be even and there would be a difference of two residues in each case. Tryptophan, particularly, and total sulfur present special problems in this connection. Other similar discrepancies are found if the molecular weight of 35,000 is used in the calculations.

One final calculation of molecular weight has been made with the following assumptions. The figures for calculated number of residues in Table III have been rounded off to the nearest *even* number (*e.g.* for glutamic acid, to 48; for leucine, 44; for proline, 16). Tryptophan residues have been taken as 4, and total cysteine and half-cystine residues as 10. Calculated molecular weights on this basis are 36,300 and 36,200 for β-lactoglobulins A and B, respectively. The data of Piez *et al.*[1] give almost identical results, slightly higher because their analyses indicate the presence of 50 glutamic acid and 14 serine residues.

DISCUSSION

After the discovery by Aschaffenburg and Drewry that two kinds of β-lactoglobulin exist in bovine milk (1) and that the presence of one form or the other in the milk of individual cows is determined by a single gene (2), further studies indicated they were very closely related chemically (14–17). However, the difference in electrophoretic mobility, which made possible the original discovery, was shown to be attributable to the presence of two more carboxyl groups in β-lactoglobulin A than in B; this was demonstrated from titration curves by Tanford and Nozaki (16) and from electrophoretic mobilities by Timasheff and Townend (17). Additional evidence for a difference in primary structure was provided by the "hybridization" experiments of Townend, Kiddy, and Timasheff (13) and by the results of preliminary experiments by Townend and Ingram on the separation of tryptic digests of β-lactoglobulins A and B by high voltage paper electrophoresis.[8]

The differences in amino acid composition reported here provide direct confirmation of the difference in carboxyl groups and, obviously, of differences in the primary structure of the two β-lactoglobulins. Although the differences in composition are undoubtedly significant, conclusions from amino acid analysis concerning molecular weight or the presence of identical halves in the β-lactoglobulin molecules must be regarded with caution. Excellent though the automated method of Spackman, Stein, and Moore may be, one cannot be certain that a protein as large as β-lactoglobulin can be analyzed with complete accuracy.

[8] R. Townend, personal communication.

Some of the difficulties that arise when the data are manipulated mathematically have already been mentioned. Another question which must not be neglected in these considerations is the purity of the proteins analyzed. The constancy of the present analyses for most of the amino acids in the three preparations is as good a criterion as any for purity, but the presence of a small percentage of impurities would be undetectable.

Finally, it should be pointed out that the finding that β-lactoglobulins A and B differ in content of four amino acids presents somewhat of a problem in view of the evidence that the occurrence of these proteins is determined by a single gene. The probability is that more than one gene is involved but further investigation in this connection is needed.

SUMMARY

β-Lactoglobulins A and B, prepared from the milks of typed cows, and β-lactoglobulin AB have been analyzed in an automatic amino acid analyzer. The genetically different forms of the protein differ significantly in content of aspartic acid, glycine, alanine, and valine. For each of these amino acids the difference is probably two residues per molecule of protein.

The new analyses of mixed β-lactoglobulin agree well with most of the generally accepted values for this protein. However, some revision of the valine, isoleucine, and lysine values may be desirable. The true tryptophan content of this protein remains uncertain.

Acknowledgment—We are grateful to Mrs. Mildred Wilensky or her help with the calculations.

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